pii: sp-00031-14 http://dx.doi.org/10.5665/sleep.4166

Male Fertility Is Reduced by Chronic Intermittent Hypoxia Mimicking Sleep Apnea in Mice

Marta Torres, MSc[1,2]; Ricardo Laguna-Barraza, MD[3]; Mireia Dalmases, MD[1,2]; Alexandra Calle, BSc[3]; Eva Pericuesta, PhD[3]; Josep M. Montserrat, MD, PhD[1,2,4]; Daniel Navajas, PhD[1,5,6]; Alfonso Gutierrez-Adan, PhD[3]; Ramon Farré, PhD[1,4,5]

[1]CIBER Enfermedades Respiratorias, Bunyola, Spain; [2]Laboratori de la Son, Servei de Pneumologia, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain; [3]INIA, Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos, Madrid; [4]Institut Investigacions Biomèdiques August Pi Sunyer, Barcelona, Spain; [5]Unitat Biofísica i Bioenginyeria, Facultat de Medicina, Universitat de Barcelona, Spain; [6]Institut Bioenginyeria Catalunya, Barcelona, Spain

Study Objectives: Obstructive sleep apnea (OSA) is characterized by intermittent hypoxia and oxidative stress. However, it is unknown whether intermittent hypoxia mimicking OSA modifies male fertility. We tested the hypothesis that male fertility is reduced by chronic intermittent hypoxia mimicking OSA in a mouse model.

Design: Case-control comparison in a murine model.

Setting: University research laboratory.

Participants: Eighteen F1 (C57BL/6xCBA) male mice.

Interventions: Mice were subjected to a pattern of periodic hypoxia (20 sec at 5% O_2 followed by 40 sec of room air) 6 h/day for 60 days or normoxia. After this period, mice performed a mating trial to determine effective fertility by assessing the number of pregnant females and fetuses.

Measurements and Results: After euthanasia, oxidative stress in testes was assessed by measuring the expression of glutathione peroxidase 1 (Gpx1) and superoxide dismutase-1 (Sod1) by reverse-transcription polymerase chain reaction. Sperm motility was determined by Integrated Semen Analysis System (ISAS). Intermittent hypoxia significantly increased testicular oxidative stress, showing a reduction in the expression of Gpx1 and Sod1 by 38.9% and 34.4%, respectively, as compared with normoxia ($P < 0.05$). Progressive sperm motility was significantly reduced from 27.0 ± 6.4% in normoxia to 12.8 ± 1.8% in the intermittent hypoxia group ($P = 0.04$). The proportion of pregnant females and number of fetuses per mating was significantly lower in the intermittent hypoxia group (0.33 ± 0.10 and 2.45 ± 0.73, respectively) than in normoxic controls (0.72 ± 0.16 and 5.80 ± 1.24, respectively).

Conclusions: These results suggest that the intermittent hypoxia associated with obstructive sleep apnea (OSA) could induce fertility reduction in male patients with this sleep breathing disorder.

Keywords: obstructive sleep apnea, hypoxia, male fertility, oxidative stress

Citation: Torres M, Laguna-Barraza R, Dalmases M, Calle A, Pericuesta E, Montserrat JM, Navajas D, Gutierrez-Adan A, Farré R. Male fertility is reduced by chronic intermittent hypoxia mimicking sleep apnea in mice. *SLEEP* 2014;37(11):1757-1765.

INTRODUCTION

Obstructive sleep apnea (OSA) is a prevalent chronic disease, particularly in men.[1,2] It is characterized by repeated episodes of total or partial collapse of the upper airway during sleep. These respiratory events cause intermittent hypoxemia, increased negative intrathoracic pressures, and sleep fragmentation. The cardiovascular,[3,4] metabolic,[5,6] neurocognitive,[7,8] and, more recently, neoplastic[9,10] consequences of OSA have been widely investigated and substantiated both at the experimental and clinical levels. Systemic and organ-specific oxidative stress triggered by intermittent hypoxia is thought to play a key role in developing these mid- and long-term consequences of OSA.[11]

In a quite different context, oxidative stress has been found to be the main cause of a growing number of cases of male infertility. Indeed, systemic oxidative stress, which is enhanced as a result of smoking, alcohol abuse, and exposure to toxins, inflammatory processes, or several chronic diseases, reduces male fertility.[12] Specifically, severe respiratory dysfunctions such as chronic obstructive pulmonary disease or pulmonary alveolar proteinosis, which are associated with systemic hypoxemia, have been related to testicular function impairment.[13] It is noteworthy that in idiopathic male infertility, which includes approximately 50% of cases,[14] higher seminal production of reactive oxygen species (ROS) and lower antioxidant capacity have been reported.[15] Moreover, there is evidence that the polyunsaturated fatty acids of the sperm membrane are very susceptible to peroxidation by ROS, and that lipid peroxidation could result in morphologic sperm alteration.[16] In fact, seminal oxidative stress assessment, either by quantifying ROS or total antioxidant capacity, is an independent predictor of male infertility[17,18] and low antioxidant capacity is related to poor sperm parameters.[19]

Given that OSA induces oxidative stress and that this challenge reduces male fertility, it could be expected that a potential consequence of OSA could lead to the reduction of fertility in male patients with this sleep breathing disorder, particularly in severe cases with hypoxic events of considerable magnitude. Although a connection between low male fertility, obesity, and OSA has been recently suggested on theoretical bases,[20] neither clinical nor experimental data are currently available to substantiate this potentially complex relationship. To test the

A commentary on this article appears in this issue on page 1731.

Submitted for publication January, 2014
Submitted in final revised form March, 2014
Accepted for publication April, 2014
Address correspondence to: Ramon Farré, PhD, Unitat de Biofísica i Bioenginyeria, Facultat de Medicina, Casanova, 143, 08036 Barcelona, Spain; Tel: +34 934024515; E-mail: rfarre@ub.edu

hypothesis that OSA-induced hypoxic events reduce male fertility, we have carried out an experimental study on a well-controlled mouse model of chronic intermittent hypoxia mimicking OSA. Specifically, we have investigated whether intermittent hypoxia induces events of hypoxia/reoxygenation in testicular tissue, enhances testicular oxidative stress, and results in reduction of sperm motility. Moreover, we have carried out a mating test to determine whether male fertility is actually decreased in mice subjected to an intermittent hypoxia pattern simulating the one observed in patients with severe OSA.

MATERIAL AND METHODS

Animals

This study, which was approved by the Ethical Committee for Animal Research of the University of Barcelona, was conducted on a total of 44 pathogen-free F1 (C57BL/6xCBA) male mice (Charles River Laboratories, Saint Germain sur L'arbresle, France). The animals were housed in standard cages and had tap water and food *ad libitum* and kept in a temperature- and light- controlled room (22-24ºC, 12L:12D).

System for Applying Chronic Intermittent Hypoxia

Chronic intermittent hypoxia was applied by a previously described setting[9] Briefly, a continuous flow of gas circulated through a box (26 cm long, 18 cm wide, 6 cm high) by means of a distribution system based in multiple orifices. A pneumatic valve placed near the inlet of the box cyclically switched from the room air entrance (40 sec) to a gas reservoir of hypoxic air at an oxygen fraction of 5% (20 sec). Therefore, intermittent hypoxia with a frequency equivalent to 60 apneas/h, which is representative of severe OSA, was applied to the mice placed in the box. To subject control mice to normoxic breathing, the animals were placed in a box identical to the one for intermittent hypoxia, but the gas reservoir of 5% O_2 air was replaced by room air. Accordingly, both groups of mice were subjected to exactly the same protocol with the only difference of breathing normoxic or intermittently hypoxic air.

Measurement of Testicular Hypoxia/Reoxygenation

In a first series of experiments we determined whether breathing the intermittent hypoxic air translated into hypoxia-reoxygenation at the testicular level. Tissue partial pressure of oxygen in the testicles (PtO_2) was measured in six anesthetized (urethane 20%, 1g/kg) 12-w-old mice. After shaving and cleaning the underlying skin of the abdomen, a transverse incision under the penis was made to expose the testicle. A second minimal incision was made in the scrotal sac and tunica to insert a fast-response Clark polarographic oxygen microelectrode pipette (OX-50, Unisense A/S, Denmark; 50 µm diameter, 90% response time < 2 sec) 3 mm below the testicular surface. When a stable baseline recording was obtained, intermittent hypoxia was applied with a nasal mask for 15 min. The time course of PtO_2 was measured by the oxygen microelectrode, connected to an amplified picoammeter (Unisense A/S, Denmark) previously calibrated in water at 100% O_2, 21% O_2 and oxygen-free solution (NaOH 0.1 M, sodium ascorbate 0.1 M) and recorded (MicOX software, Unisense A/S). Arterial oxygen saturation (SaO_2) was also measured and recorded by pulse oximetry (MouseOx Plus, Starr Life Sciences Corp).[21]

Effect of Intermittent Hypoxia on Testicular Oxidative Stress and Sperm Motility

A second series of experiments was aimed at assessing the effect of 30-day exposure to intermittent hypoxia into oxidative stress in testicular tissue and sperm motility in young animals. To this end, 20 mice (12 w old) were randomly separated into two groups (control and intermittent hypoxia; n = 10 each) and weighted (25.5 ± 0.6 g and 26.0 ± 0.3, mean ± standard error (m ± SE) in the normoxia and intermittent hypoxia groups, respectively; P = 0.53, *t*-test). The animals were placed into the experimental cage and subjected to intermittent hypoxia or normoxia for 6 h per day during the light period (10:00–16:00), which corresponds to the sleep time in rodents. After 30 days under intermittent hypoxia or normoxia, the mice were euthanized by exsanguination. Sperm were released from the epididymis to carry out a conventional seminogram to measure total motility and progressive motility values. Viability and sperm counts were also analyzed. To evaluate spermatozoa motility and progressive motility, testis, epididymis, and vas deferens were removed. Fat and veins dissected away from the vas deferens to avoid contamination. Moreover, the testicles were excised and stored at -80 ºC to determine glutathione peroxidase 1 (Gpx1), superoxide dismutase 1 (Sod1) and catalase (Cat) by reverse-transcription polymerase chain reaction (RT-PCR).

Sperm Motility Measured by Seminogram

Sperm were placed into a 35 mm-well containing 500 µL of M2 medium (Sigma-Aldrich, St, Louis, MO, US) by exerting soft pressure from the cauda epididymis to the end of the vas deferens with the help of watchmaker's tweezers. The sperm sample was incubated at 37ºC for 15 min until the sperm were homogeneously distributed in the M2 drop. A sample of 25 µL from the surface of the drop (swim-up) was placed on a microscope slide to obtain quantitative sperm motility variables. Sperm motility and progressive sperm motility measurements were analyzed using an Integrated Semen Analysis System (ISAS, Proiser, Valencia, Spain).[22] The parameters used for this analysis were smoothed path velocity, track velocity, straightness (ratio of straight line velocity/average path velocity, VSL/VAP), and amplitude of lateral head placement, based on total motility, progressive motility, and speed (static, medium and slow sperm cells). For sperm counts, a sample of sperm was diluted 1/10 in milli-Q water and 10 µL were placed in a Bürker chamber to obtain sperm cells concentrations (million spermatozoa/mL) using a standard procedure. Viability was determined considering percentages of live and dead sperm cells using a live-cell nucleic acid stain, SYBR-14, in combination with the conventional dead-cell nucleic acid stain, propidium iodide[23] using the live/dead sperm viability kit (Molecular Probes, Eugene, OR). Briefly, 0.8 mL of 20 mM SYBR-14 working solution and 1.2 ml of 2.4 mM propidium iodide working solution were added to 50 mL of the sperm suspension (2–3 × 10^6 sperm cells/mL) and incubated at 37ºC for 15 min. After 15 min, 20 mL of the sperm suspension were loaded on a glass slide, covered with a coverslip, and immediately observed under a fluorescent microscope equipped with

appropriate filters. SYBR-14 stains green the nucleus of live sperm, whereas dead or membrane-damaged spermatozoa are stained red by the propidium iodide.

Assessment of Oxidative Stress

The techniques for analysis of marker gene expression by RT-PCR have been described in detail previously.[24] Total RNA was extracted from testis of mice under intermittent hypoxia or normoxia using Trizol Reagent (Invitrogen, Life Technologies, Madrid, Spain) following the manufacturer's instructions with some modifications. Immediately, real-time reaction was performed according to the manufacturer's instructions (Gibco-BRL, Grand Island, NY); 0.2 mM oligo (dT), 0.5 mM of random primers were added to messenger RNA (mRNA) extraction and heated 5 min at 70ºC. After that, heat-denatured (65°C, 2 min) and reverse-transcribed at 42 °C for 60 min, inactivation at 70ºC 10 min in final volume of 40 µL containing 0.5 mM of each dNTP, M-MLV RT (0.5 µL), RNasin (0.2 µL) and 10× M-MLV RT buffer with 8 mM Dithiothreitol (DTT) was performed. The quantification of all mRNA transcripts was performed by real-time quantitative (q) RT-PCR using a Rotor-gene 6000 Real Time CyclerTM (Corbett Re- search, Sydney, Australia) and SYBR Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific fluorescent dye. PCR was performed by adding a 2-µL aliquot of each sample to the PCR mix (Quantimix Easy Sig Kit, Biotools) containing the specific primers to amplify Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping (5′-AGGTCGGTGTGAACGGATTTG and 5′-TGTAGACCATGTAGTTGAGGTCA, glutathione peroxidase 1 (Gpx1) (5′-GCAACCAGTTTGGGCATCA and 5′-CTCGCACTTTTCGAAGAGCATA), and superoxide dismutase 1 (Sod1) (5′-GTGCAAGGCACCATCCACTTCG and 5′-CACCATCGTGCGGCCAATGATG and catalase (Cat) (5′-CCGACCAGGGCATCAAAA and 5′-GAGGCCATAATCCGGATCTTC). The PCR protocol included an initial step of 94°C (2 min), followed by 35 cycles of 94°C (15 sec), 56°C (30 sec), and 72°C (30 sec). The melting protocol consisted of holding at 40°C for 60 sec and then heating from 50°C to 94°C, holding at each temperature for 5 sec while monitoring fluorescence. For qRT-PCR, six (hypoxia) and five (control) groups of complementary DNA were used with two repetitions for all genes of interest. As negative controls, we always supplied sample for water. The comparative cycle threshold (CT) method was used to quantify expression levels. Quantification was normalized to the endogenous control GAPDH. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the GAPDH CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$. Data on mRNA expression were analyzed using the Prism 5 (GraphPad Software, La Jolla, CA, US) software package.

Effect of Intermittent Hypoxia on Male Fertility

The third series of experiments was carried out to determine the actual effect of a long period of intermittent hypoxia on the fertility of middle-aged male mice. A conventional mating test was conducted.[22] The same protocol of intermittent hypoxia (and normoxia in controls) was applied to a group of 18 old mice (age: 12 mo) for 60 days. The animals were randomly separated into two groups (8 and 10 mice for the normoxia and intermittent hypoxia groups, respectively) and weighted (35.0 ± 2.2 g and 36.8 ± 2.3 g, respectively; P = 0.57, *t*-test). After the 2-mo exposure to intermittent hypoxia or normoxia, all mice performed an *in vivo* fertility test. Three virgin female CD1 mice of 8-12 w of age were partnered with each male on 5 consecutive days. Every day during cohabitation, females were examined for vaginal plugs as evidence of mating. At the end of the mating test, the males were euthanized by cervical dislocation and the epididymis and testis were excised to assess testicular oxidative stress and sperm motility as described previously. On gestation day 14, females were euthanized using CO_2 and the variables percentage of pregnant females, resorptions per litter, and litter size were recorded. Live fetuses were euthanized after examination.

Data Analysis

The results are presented as mean ± SE. Comparisons between baseline, maximum, and minimum values of PtO_2 or SaO_2 were performed by means of paired *t*-tests. Differences in mRNA expression were analyzed by one-way repeated-measures analysis of variance with arcsine data transformation and significance determined by using the Holm-Sidak *post hoc* test. Comparison between groups of data from seminograms and mating tests were performed by means of *t*-tests or the Mann-Whitney rank-sum test as required. A P value ≤ 0.05 was considered statistically significant.

RESULTS

As a result of breathing intermittent hypoxic air, the mice experienced cyclic changes in SaO_2 ranging from maxima of 95.4 ± 0.1% (similar to baseline values) to minima of 62.3 ± 3.5% (P < 0.001), thereby mimicking those observed in patients with severe OSA (Figure 1, top). Measurement of local oxygenation at the testes indicated that their tissues were subjected to considerable oscillations of oxygen partial pressure ranging from 11.1 ± 1.6 mmHg (similar to baseline figures) down to 3.6 ± 1.5 mmHg (P < 0.001) (Figure 1, bottom), indicating that the testicles were actually subjected to fast cyclic events of hypoxia-reoxygenation.

After 30 days under intermittent hypoxia, increased oxidative stress was detected at the testicular level of the young mice since the expressions of two main antioxidant enzymes (Gpx1 and Sod1) were significantly reduced by 37% and 57%, respectively, as compared with controls (P < 0.05) (Figure 2, top). No significant differences were observed in the expression of catalase. The markers of oxidative stress experienced a similar behavior in the middle-aged mice breathing hypoxic air for 60 days, showing a reduction in the expressions of Gpx1 and Sod1 of 39% and 34%, respectively (P < 0.05) (Figure 2, bottom).

Progressive sperm motility was significantly reduced (P < 0.04) by intermittent hypoxia in the young males subjected

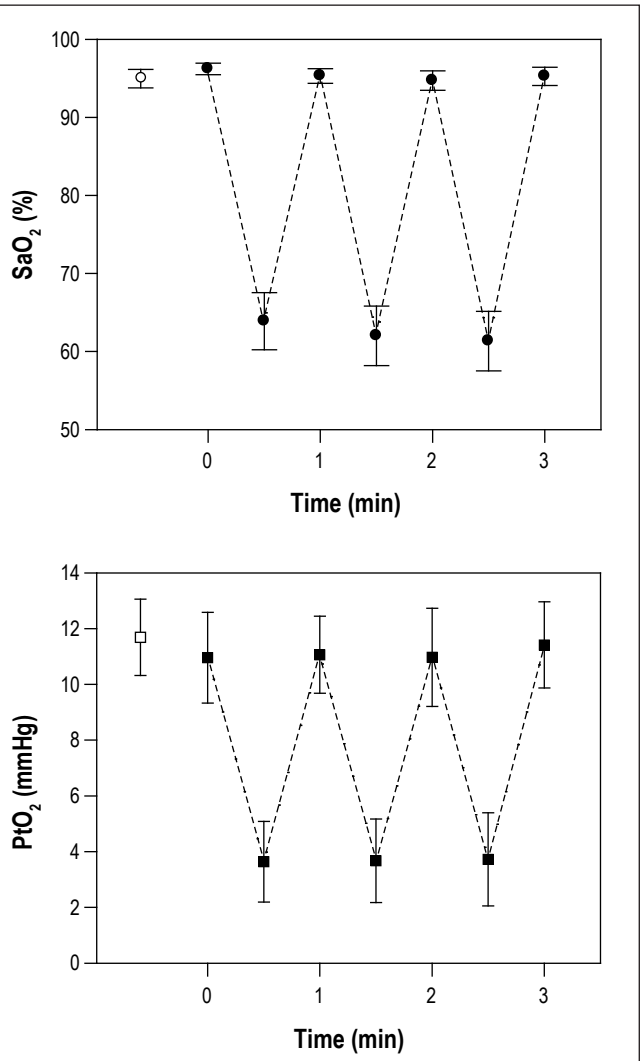

Figure 1—Time course of arterial oxygen saturation (SaO_2) (top) and testicular oxygen partial pressure (PtO_2) (bottom). Baseline SaO_2 value, represented by the open circle (94.9 ± 1.9%), was similar to maximal values (95.4 ± 0.1%) and significantly different from the minima of 62.3 ± 3.5% (P < 0.001). Testicular PtO_2 oscillated from 11.1 ± 1.6 mmHg (similar to baseline value: 11.7 ± 1.4, represented by the open square) down to 3.6 ± 1.5 mmHg (P < 0.001).

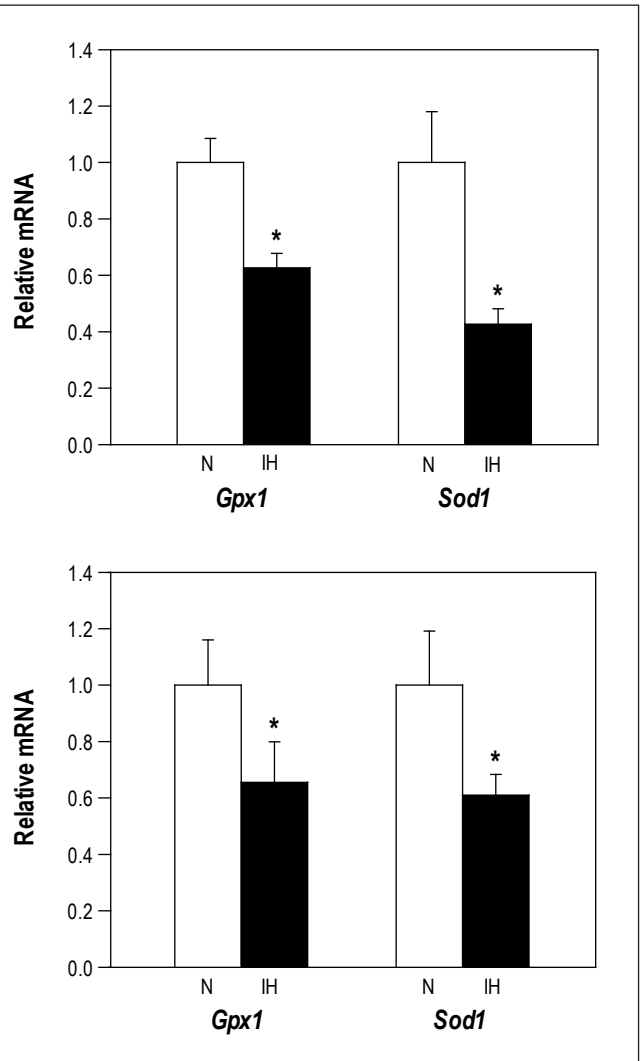

Figure 2—Relative gene expression of antioxidant enzymes glutathione peroxidase 1 (Gpx1) and superoxide dismutase-1 (Sod1) of young (top) and middle-aged mice (bottom). Gpx1 and Sod1 expression in young mice subjected to 30-day intermittent hypoxia was reduced by 37% and 57%, respectively (P < 0.05). In middle-aged mice subjected to 60-day intermittent hypoxia the reduction was 39% and 34%, respectively (P < 0.05). IH, intermittent hypoxia; mRNA, messenger RNA; N, normoxia.

to 30 days of intermittent hypoxia: from 31.5 ± 3.5% in the normoxic group to 22.9 ± 1.8% (Figure 3, top). No significant differences were observed in the other sperm parameters (total sperm concentration, viability, and apoptosis; data not shown). The decrease in progressive sperm motility was even greater in the middle-aged mice subjected to 60 days of intermittent hypoxia in comparison with their normoxic controls: from 27.0 ± 6.4% in normoxia to 12.8 ± 1.8% (P = 0.045) (Figure 3, bottom).

Intermittent hypoxia caused a marked decrease in actual male fertility. The number of pregnant females per mating was significantly higher (P = 0.04) in the normoxic group (0.72 ± 0.16) than in the intermittent hypoxia group (0.33 ± 0.10) (Figure 4, top). Moreover, the number of fetuses per mating was reduced in the intermittent hypoxia group (2.45 ± 0.73) when compared to normoxic controls (5.80 ± 1.24) (P = 0.02) (Figure 4, center). Consistently, the average number of fetuses per litter was similar in both groups (8.10 ± 0.19 in controls and 7.42 ± 0.65 in the intermittent hypoxia group), indicating that males capable of impregnating females have sperm of sufficient quality to produce normal litter sizes. There were no differences in the number of resorptions per litter. As shown in Figure 4 (bottom), the observed decrease in male fertility (both in number of pregnant females and fetuses) was not caused by a reduction in the number of mating intercourses, because the number of copulatory plugs per female was similar in the normoxia and intermittent hypoxia groups: 0.62 ± 0.16 and 0.70 ± 0.09, respectively.

DISCUSSION

This experimental study presents novel data supporting the notion that a pattern of chronic intermittent hypoxia mimicking OSA actually reduces male fertility. Interestingly, we found alteration in sperm motility even in the experimental series where it was less expected. This was in the youngest mice in whom

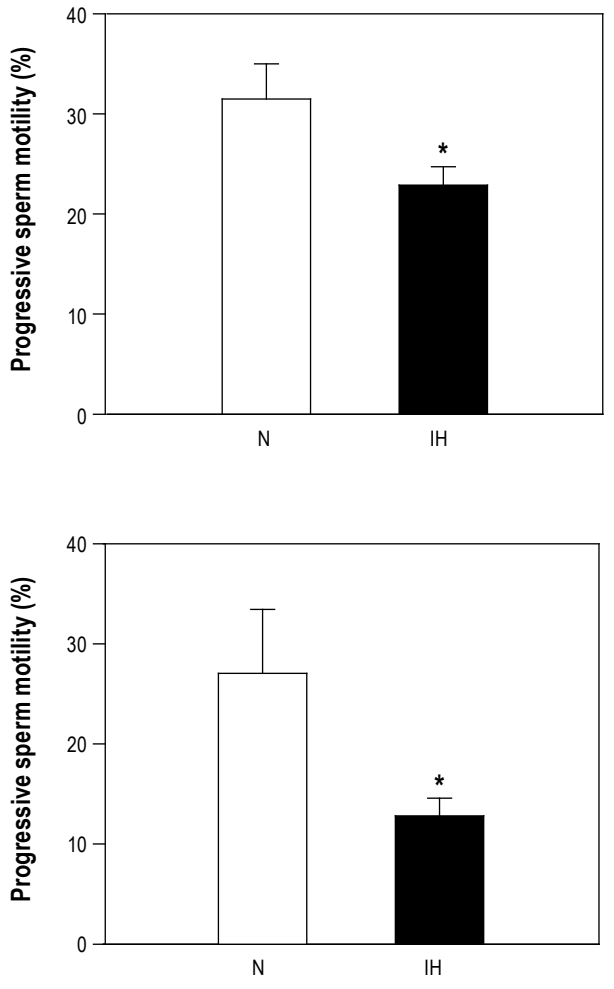

Figure 3—Progressive sperm motility (%) of young (top) and middle-aged mice (bottom). Progressive sperm motility in young mice was decreased after intermittent hypoxia from 31.5 ± 3.5% in normoxia to 22.9 ± 1.8% in the 30-day intermittent hypoxia group (P < 0.04). In middle-aged mice, progressive sperm motility was reduced from 27.0 ± 6.4% in controls to 12.8 ± 1.8% in the 60-day intermittent hypoxia group (P = 0.04). N, normoxia; IH, intermittent hypoxia.

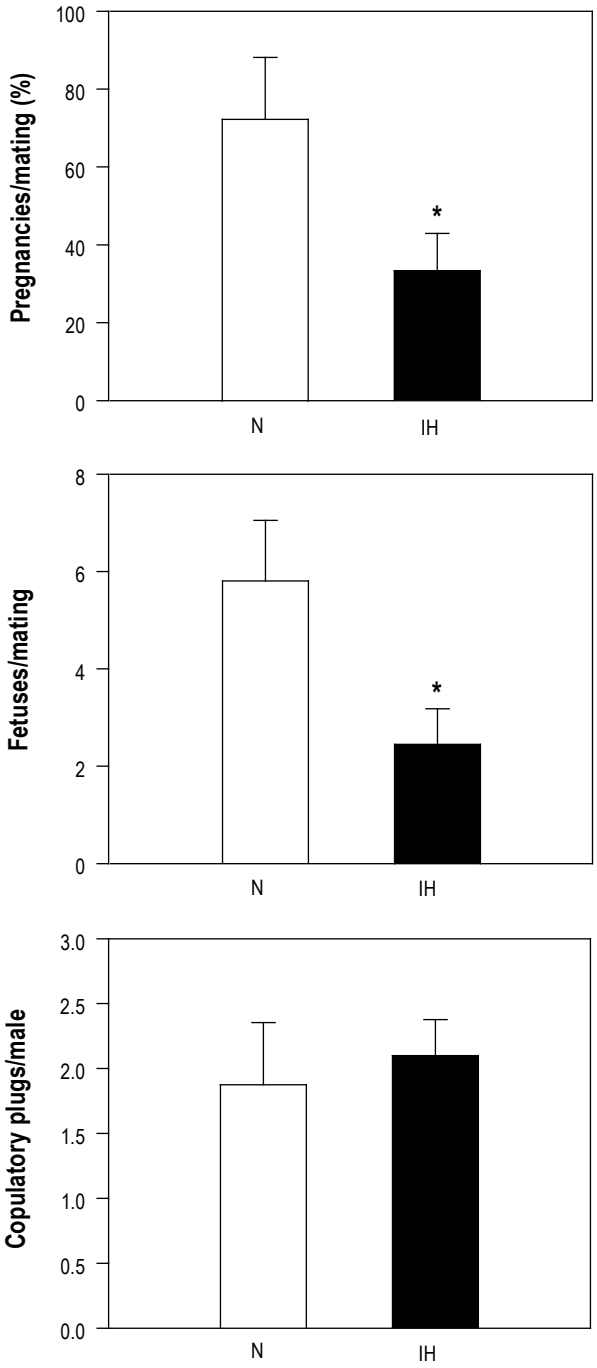

Figure 4—Pregnant females per mating (%) (top), average of fetuses per mating (middle), and copulatory plugs per male (bottom) after the mating trial. The number of pregnant females per mating was higher (P = 0.04) in the control group (0.72 ± 0.16) in comparison with the intermittent hypoxia group (0.33 ± 0.10). The number of fetuses per mating was reduced in the intermittent hypoxia group (2.45 ± 0.73) in comparison with controls (5.80 ± 1.24) (P = 0.025). No differences were found in the number of copulatory plugs per female: 0.62 ± 0.16 in normoxia and 0.70 ± 0.09 in intermittent hypoxia group. N, normoxia; IH, intermittent hypoxia.

sperm quality is unaffected by aging,[25] subjected to only 30 days of intermittent hypoxia, which is slightly below the ~40-day duration of spermatogenesis in mice.[26] Moreover, the most relevant result was the decrease in offspring observed in the animals that more realistically mimicked the clinical conditions of patients with OSA: middle-aged mice subjected to a long duration, chronic, intermittent hypoxia.

Previous experimental studies on the effects of hypoxia on sperm quality were carried out under continuous hypoxia[27,28] or when the animals were subjected to alternate periods of normoxia and hypoxia, lasting several days each, to simulate processes of cyclic adaptation to low and high altitudes.[29–31] These studies reported testicular/sperm oxidative stress and other histological/anatomical alterations induced by low-oxygen concentration in the air. However, data obtained under constant or extremely low-frequency alternating hypoxia did not provide information on the potential effect of the high-frequency intermittent hypoxia characterizing OSA on male fertility. Therefore, the experimental approach in the current study was specifically addressed to this end. Indeed, the hypoxic breathing pattern imposed to the mice was of considerable, but in no way exceptional, severity, being similar to the typical

hypoxic paradigm employed in experimental OSA research.[6,9,32] Moreover, to better characterize the entire process involved we investigated variables at different levels in the pathophysiologic cascade. First, we documented that intermittent hypoxia actually translated into high-frequency hypoxia/reoxygenation events in the testicular tissue (Figure 1). Second, associate oxidative stress at the testes was assessed by conventional biomarkers (Figure 2). Third, we found a subsequent loss of sperm progressive motility (Figure 3). And finally, actual reduction in male fertility was documented by mating tests (Figure 4).

Local occurrence of hypoxia/reoxygenation events at the testicular level, a direct consequence of the intermittently hypoxemic blood supply, should be considered the main cause of the subsequent spermatic alterations observed. Testicular PtO_2 was measured by means of an electrochemical microcatheter providing excellent resolution for this application: small sample area observed and fast time response (50-μm diameter, 90% response time < 2 sec).[33] In fact, this tool has been previously used to measure PtO_2 in other tissues (brain, muscle, fat, liver) in rodent models of OSA.[21,34] Interestingly, the ~12 mmHg baseline value we obtained in mice testes agrees with previous data in other species such as rat, rabbit, sheep, and dog.[35–37] This figure of testicular PtO_2 is clearly below the oxygenation levels characterizing other tissues: in fact, different reports indicate that PtO_2 at the testes is around half the value in most relevant tissues.[38] The fact that the testes work at a relatively hypoxic level has been attributed to a high oxygen extraction because of the metabolic demands of spermatogenesis, and because the testes have reduced ability to increase blood flow.[39] Accordingly, the testes could be particularly sensitive to the intermittently O_2-desaturated blood supply typical in OSA, as reflected by the considerable magnitude of the PtO_2 swings caused by recurrent hypoxia (~4 mmHg nadir; Figure 1) with potential important consequences on the process of spermatogenesis and hence fertility.

A wide variety of endogenous and exogenous factors are known to generate a state of oxidative stress in testis (e.g., heat stress, varicocele, diabetes, infections, hypoxia).[40] It has been reported that the mechanism by which continuous hypoxia affect at the testicular level is probably mediated by inducing oxidative stress owing to an increase in reactive oxygen species formation and impairment in the oxidative defense mechanisms, contributing to damage in the spermatogenic cells and apoptosis.[40] During this process up-regulation of hypoxia and oxidative stress related genes are present.[40] However, in our intermittent hypoxia experiment, two of the three oxidative stress markers analyzed (Gpx1 and Sod1) were underexpressed in the testes of intermittent hypoxia-treated males, indicating that the oxidative protection is compromised in a different way to the continuous hypoxia. Unlike continuous hypoxia that produce germ cell damage and apoptosis, our intermittent hypoxia model only produces a reduction on progressive sperm motility and fertility.

To reduce the generation of ROS, testes have developed a complex antioxidant system, and the induction of oxidative stress in the testes precipitates a response characterized by induction of mRNA species for SOD, GPx, and glutathione-S-transferase (GST) activities.[41] They produce a rapid conversion of superoxide anion (O_2^{-}.) to hydrogen peroxide (H_2O_2) in the presence of SOD in order to prevent the former from participating in the formation of highly pernicious hydroxyl radicals. The H_2O_2 generated in this manner is a powerful membrane permeant oxidant and in order to prevent the induction of oxidative damage to lipids, proteins, and DNA, they have to be rapidly eliminated from the cell. The elimination of H_2O_2 is either effected by catalase or glutathione peroxidase, with the latter predominating in the case of the testes.[40,42] We think that in our experiment the level of catalase could not be affected because of its limited importance in the testis.

The reduction on Gpx1 and Sod1 expression could be related to the reduction on both progressive sperm motility and fertility (reduction of pregnant female). However, the average number of fetuses per litter was not affected in the intermittent hypoxia group, indicating that males capable of impregnating females have sperm DNA of sufficient quality to produce normal embryos. In agreement with our results, it has been reported that Sod1 knockout mice (*Sod1*-KO) sperm has a lower fertilizing ability than wild-type sperm *in vitro*; however, once fertilized, the embryos developed normally to the blastocyst stage.[43] Interestingly, progressive sperm motility of *Sod1*-KO mice declined during sperm incubation in a drop medium, and lipid peroxidation products adversely increased in *Sod1*-KO sperm.[43] Recently, it has been also published that the *Sod1*-KO mice have zero *in vivo* fertilization success in sperm competition trials that pit them against wild-type males and are almost completely infertile when mated singly with females of a different genotype.[44] Moreover, in agreement with our intermittent hypoxia mice, *Sod1*-KO mice did not differ in their mating behavior. We suggest that the oxidative insult caused by the intermittent hypoxia in the testis is caused by reduction in some antioxidant enzymes that result in decreased sperm progressive motility and sperm-fertilizing ability without affecting other reproductive characteristics such as sperm production, sperm DNA quality, or matting behaviors.

Data in this study strongly suggest that the local effect of intermittent hypoxia—via oxidative stress—in the tissues directly involved in spermatogenesis could reduce male fertility. However, it could also be possible that intermittent hypoxia affects local or systemic mediators that play a role in modulating fertility. Indeed, testosterone and follicle-stimulating hormone are the two major endocrine signals that act in the testis to regulate spermatogenesis efficiency. Testosterone is produced by Leydig cells present in the interstitial space of the testis between the seminiferous tubules and then diffuses into the tubules. Because testosterone is produced locally by the Leydig cells, testosterone levels in the testes of men and rodents are 25–125-fold higher than that present in serum.[45] However, the physiological necessity for high levels of testosterone in the testis is not well understood. Although these high intratesticular testosterone levels may be required to quantitatively maintain maximum spermatogenic potential, qualitatively normal spermatogenesis can be maintained with much lower intratesticular concentrations[46] and the bioavailable testosterone in the testis greatly exceeds the 1 nM that is required for regulation of gene expression via androgen receptor (AR) binding to gene promoters.[47] In addition to the classic model of testosterone and AR actions that were centered on the Sertoli cell, testosterone actions in other cell types including peritubular myoid cell

and vascular smooth muscle cells are increasingly being found to affect processes that occur in the seminiferous tubules.[48] Nevertheless, how intermittent hypoxia *per se* may affect testosterone levels in OSA remains unclear from clinical data because of confounding factors such as age, obesity, or sleep disruption. Contrary to data clearly demonstrating a decrease in testosterone concentrations in sleep deprived male rats,[49,50] the evidence available from animal research on the effect of hypoxia (continuous or intermittent) on sexual hormones is not conclusive because increase,[51] decrease,[52] and no change[53] in serum testosterone concentration after hypoxia application have been reported. Therefore, the lack of data on the specific role of testosterone on the fertility of males subjected to intermittent hypoxia, a limitation of the current study, deserves further investigation.

Although OSA has been reported to induce alterations in male sexual function including decreased libido and erectile dysfunction in both animals[53] and in patients,[54–57] potentially modulated by testosterone changes,[58] no data are available on fertility. In addition to recurrent hypoxia-reoxygenation events, male patients with OSA experience other challenges that could negatively affect sperm quality and fertility. One of these potential challenges is directly linked to the nocturnal events in OSA: disruption of sleep architecture caused by microarousals accompanying most of the upper airway obstructive events. However, the information currently available on the effects of sleep alterations on male fertility is scarce. Whereas no significant alterations in sperm parameters were found after application of chronic sleep restriction in mice,[59] a recent study showed an association between self-reported sleep disturbances with poor sperm quality in young men from the general population.[60] However, one of the most important concomitant factors potentially affecting male fertility in OSA is obesity because these two disorders are very frequently associated.[61] It has been reported that obesity *per se* is a factor that slightly but significantly reduces fertility in otherwise healthy men.[62,63] Such an effect of increased body mass index (BMI) on male fertility has been attributed to several potential reasons. First, obese men have abnormal reproductive hormone profiles that may impair spermatogenesis.[64] Second, testicular heat stress may play a role in obesity-related impaired spermatogenesis because the effects of sitting posture increased in sedentarism—and fat accumulation in the areas surrounding the testis.[65] Given that obesity induces an increase in oxidative stress at systemic level,[66] a third mechanism by which increased BMI may impair male fertility is oxidative stress-initiated damage to sperm.[67] In fact, an increased BMI was associated with a moderate augmentation in sperm DNA damage.[68] Interestingly, oxidative stress independently induced by both excessive BMI and intermittent hypoxemia could be synergistically deleterious for the fertility of a substantial part of men suffering from OSA.

In conclusion, this experimental study provides proof of concept on the negative effects of high-frequency intermittent hypoxia on sperm function. Subsequent research, both in animal models and in patients, is required to substantiate whether male patients with OSA would suffer from reduced fertility. On the one hand, animal studies will be helpful in clarifying the hypoxic dose-response effects or in assessing whether treatments with antioxidant drugs could reduce the deleterious role of intermittent hypoxia on reproductive health.[69] Moreover, animal research combining intermittent hypoxia with experimental models of other comorbidities typical of OSA (e.g., application of experimental sleep fragmentation, and using obese, diabetic, or hypertensive mice) could be useful to explore potential synergistic effects. On the other hand, patient studies should shed light on a potential association between OSA and altered male fertility. Specifically, to determine whether sperm quality assessed by conventional seminogram is reduced in patients with OSA and, if this is the case, whether the conventional index of nocturnal oxygen desaturation (after correcting by other confounding factors such as obesity or sleep disruption indices) plays a main role, and to explore whether treatment with continuous positive airway pressure would improve fertility. Finally, it is noteworthy that the number of patients potentially affected by fertility problems associated with sleep breathing disturbances is expected to increase because the rising overlap between the time windows of paternal age and OSA prevalence. Indeed, whereas fatherhood shows a sustained tendency to be delayed,[70,71] OSA will be more prevalent in young patients given the obesity epidemic.[72,73] Specifically, current prevalence estimates of moderate to severe sleep disordered breathing in different general populations for the age interval more relevant for male fertility range from 10% (age 30-49 y)[1] to 16% (age 30-39 y) and 35% (age 40-49 y).[2]

ACKNOWLEDGMENTS

The authors thank Mr. Miguel A. Rodríguez and Mrs. Maeba Polo for their excellent technical assistance.

DISCLOSURE STATEMENT

This was not an industry supported study. This work was supported in part by the Spanish Ministry of Economy and Competitiveness (SAF2011-22576; AGL2012-39652-C02-01; FIS-PI11/00089). The authors have indicated no financial conflicts of interest.

REFERENCES

1. Peppard PE, Young T, Barnet JH, Palta M, Hagen EW, Hla KM. Increased prevalence of sleep-disordered breathing in adults. Am J Epidemiol 2013;177:1006-14.
2. Tufik S, Santos-Silva R, Taddei JA, Bittencourt LRA. Obstructive sleep apnea syndrome in the Sao Paulo Epidemiologic Sleep Study. Sleep Med 2010;11:441–6.
3. Almendros I, Farré R, Torres M, et al. Early and mid-term effects of obstructive apneas in myocardial injury and inflammation. Sleep Med. 2011;12:1037–40.
4. Martínez-García MA, Capote F, Campos-Rodríguez F, et al; Spanish Sleep Network. Effect of CPAP on blood pressure in patients with obstructive sleep apnea and resistant hypertension: the HIPARCO randomized clinical trial. JAMA 2013;310:2407–15.
5. Trzepizur W, Le Vaillant M, Meslier N, et al. Independent association between nocturnal intermittent hypoxemia and metabolic dyslipidemia. Chest 2013;143:1584–9.
6. Drager LF, Yao Q, Hernandez KL, Shin MK, et al. Chronic intermittent hypoxia induces atherosclerosis via activation of adipose angiopoietin-like 4. Am J Respir Crit Care Med 2013;188:240–8.
7. Torelli F, Moscufo N, Garreffa G, et al. Cognitive profile and brain morphological changes in obstructive sleep apnea. Neuroimage 2011; 54:787–93.
8. Row BW, Kheirandish L, Cheng Y, Rowell PP, Gozal D. Impaired spatial working memory and altered choline acetyltransferase (CHAT) immunoreactivity and nicotinic receptor binding in rats exposed to intermittent hypoxia during sleep. Behav Brain Res 2007;177:308–14.

9. Almendros I, Montserrat JM, Torres M,, Duran-Cantolla J, Navajas D, Farré R. Intermittent hypoxia enhances cancer progression in a mouse model of sleep apnoea. Eur Respir J 2012;39:215–7.
10. Campos-Rodriguez F, Martinez-Garcia MA, Martinez M, et al; Spanish Sleep Network. Association between obstructive sleep apnea and cancer incidence in a large multicenter Spanish cohort. Am J Respir Crit Care Med 2013;187:99–105.
11. Lavie L. Oxidative stress--a unifying paradigm in obstructive sleep apnea and comorbidities. Prog Cardiovasc Dis 2009;51:303–12.
12. Tremellen K. Oxidative stress and male infertility-a clinical perspective. Hum Reprod Update 2008;14:243–58.
13. Bomhard EM, Gelbke HP. Hypoxaemia affects male reproduction: a case study of how to differentiate between primary and secondary hypoxic testicular toxicity due to chemical exposure. Arch Toxicol 2013;87:1201–18.
14. Iammarrone E, Balet R, Lower AM, Gillott C, Grudzinskas JG. Male infertility. Best Pract Res Clin Obstet Gynaecol 2003;17:211–29.
15. Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ Jr, Agarwal A. Oxidative stress in normospermic men undergoing infertility evaluation. J Androl 2001;22:316–22.
16. Rao B, Soufir JC, Martin M, David G. Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. Gamete Res 1989;24:127–34.
17. Agarwal A, Sharma RK, Nallella KP, Thomas AJ Jr, Alvarez JG, Sikka SC. Reactive oxygen species as an independent marker of male factor infertility. Fertil Steril 2006;86:878–85.
18. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. The reactive oxygen species total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999;14:2801–7.
19. Pahune PP, Choudhari AR, Muley PA. The total antioxidant power of semen and its correlation with the fertility potential of human male subjects. J Clin Diagn Res 2013;7:991–5.
20. Hammoud AO, Carrell DT, Gibson M, Peterson CM, Meikle AW. Updates on the relation of weight excess and reproductive function in men: sleep apnea as a new area of interest. Asian J Androl 2012;14:77–81.
21. Almendros I, Farré R, Planas AM, et al. Tissue oxygenation in brain, muscle, and fat in a rat model of sleep apnea: differential effect of obstructive apneas and intermittent hypoxia. Sleep 2011;34:1127–33.
22. Calle A, Miranda A, Fernandez-Gonzalez R, Pericuesta E, Laguna R, Gutierrez-Adan A. Male mice produced by in vitro culture have reduced fertility and transmit organomegaly and glucose intolerance to their male offspring. Biol Reprod 2012;87:34.
23. Perez-Crespo M, Pintado B, Gutierrez-Adan A. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. Mol Reprod Dev 2008;75:40–7.
24. Bermejo-Alvarez P, Rizos D, Lonergan P, Gutierrez-Adan A. Transcriptional sexual dimorphism in elongating bovine embryos: implications for XCI and sex determination genes. Reproduction 2011;141:801–8.
25. Katz-Jaffe MG, Parks J, McCallie B, Schoolcraft WB. Aging sperm negatively impacts in vivo and in vitro reproduction: a longitudinal murine study. Fertil Steril 2013;100:262-8.e1-2.
26. Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat 1956;99:507–16.
27. Liao W, Cai M, Chen J, Huang J, Liu F, Jiang C, Gao Y. Hypobaric hypoxia causes deleterious effects on spermatogenesis in rats. Reproduction 2010;139:1031–8.
28. Madrid D, Reyes JG, Hernández B, et al. Effect of normobaric hypoxia on the testis in a murine model. Andrologia 2013;45:332–8.
29. Farias JG, Bustos-Obregón E, Orellana R, Bucarey JL, Quiroz E, Reyes JG. Effects of chronic hypobaric hypoxia on testis histology and round spermatid oxidative metabolism. Andrologia 2005;37:47–52.
30. Cikutovic M, Fuentes N, Bustos-Obregón E. Effect of intermittent hypoxia on the reproduction of rats exposed to high altitude in the Chilean Altiplano. High Alt Med Biol 2009;10:357–63.
31. Farias JG, Puebla M, Acevedo A, et al. Oxidative stress in rat testis and epididymis under intermittent hypobaric hypoxia: protective role of ascorbate supplementation. J Androl 2010;31:314–21.
32. Lee EJ, Woodske ME, Zou B, O'Donnell CP. Dynamic arterial blood gas analysis in conscious, unrestrained C57BL/6J mice during exposure to intermittent hypoxia. J Appl Physiol 2009;107:290–4.
33. Almendros I, Montserrat JM, Torres M, González C, Navajas D, Farré R. Changes in oxygen partial pressure of brain tissue in an animal model of obstructive apnea. Respir Res 2010;11:3.
34. Reinke C, Bevans-Fonti S, Drager LF, Shin MK, Polotsky VY. Effects of different acute hypoxic regimens on tissue oxygen profiles and metabolic outcomes. J Appl Physiol 2011;111:881–90.
35. Free MJ, Schluntz GA, Jaffe RA. Respiratory gas tensions in tissues of the male rat reproductive tract. Biol Reprod 1976;14:481–8.
36. Lysiak JJ, Nguyen QA, Turner TT. Fluctuations in rat testicular interstitial oxygen tensions are linked to testicular vasomotion: persistence after repair of torsion. Biol Reprod 2000;63:1383–9.
37. Cross BA, Silver IA. Neurovascular control of oxygen tension in the testis and epididymis. J Reprod Fertil 1962;3:377–95.
38. Klotz T, Vorreuther R, Heidenreich A, Zumbe J, Engelmann U. Testicular tissue oxygen pressure. J Urol 1996;155:1488–91.
39. Setchell BP. The Mammalian Testis. London: Elek Books, Ltd, 1978:300.
40. Reyes JG, Farias JG, Henriquez-Olavarrieta S, et al. The hypoxic testicle: physiology and pathophysiology. Oxid Med Cell Longev 2012; 2012:929285.
41. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 2008;1:15–24.
42. Peltola V, Huhtaniemi I, Ahotupa M. Antioxidant enzyme activity in the maturing rat testis. J Androl 1992;13:450–5.
43. Tsunoda S, Kawano N, Miyado K, Kimura N, Fujii J. Impaired fertilizing ability of superoxide dismutase 1-deficient mouse sperm during in vitro fertilization. Biol Reprod 2012;87:121.
44. Garratt M, Bathgate R, de Graaf SP, Brooks RC. Copper-zinc superoxide dismutase deficiency impairs sperm motility and in vivo fertility. Reproduction 2013;146:297–304.
45. Comhaire FH, Vermeulen A. Testosterone concentration in the fluids of seminiferous tubules, the interstitium and the rete testis of the rat. J Endocrinol 1976;70:229–35.
46. Walker WH. Non-classical actions of testosterone and spermatogenesis. Philos Trans R Soc Lond B Biol Sci 2010;365:1557–69.
47. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451–86.
48. Chang C, Lee SO, Wang RS, Yeh S, Chang TM. Androgen receptor (AR) physiological roles in male and female reproductive systems: lessons learned from AR-knockout mice lacking AR in selective cells. Biol Reprod 2013;89:21.
49. Andersen ML, Bignotto M, Tufik S. Facilitation of ejaculation after methamphetamine administration in paradoxical sleep deprived rats. Brain Res 2003;978:31–7.
50. Andersen ML, Martins RC, Alvarenga TA, Antunes IB, Papale LA, Tufik S. Progesterone reduces erectile dysfunction in sleep-deprived spontaneously hypertensive rats. Reprod Biol Endocrinol 2007;5:7.
51. Hwang GS, Chen ST, Chen TJ, Wang SW. Effects of hypoxia on testosterone release in rat Leydig cells. Am J Physiol Endocrinol Metab 2009;297:E1039-45.
52. Liu GL, Yu F, Dai DZ, Zhang GL, Zhang C, Dai Y. Endoplasmic reticulum stress mediating downregulated StAR and 3-beta-HSD and low plasma testosterone caused by hypoxia is attenuated by CPU86017-RS and nifedipine. J Biomed Sci 2012;19:4.
53. Soukhova-O'Hare GK, Shah ZA, Lei Z, Nozdrachev AD, Rao CV, Gozal D. Erectile dysfunction in a murine model of sleep apnea. Am J Respir Crit Care Med 2008;178:644–50.
54. Luboshitzky R, Aviv A, Hefetz A, et al. Decreased pituitary-gonadal secretion in men with obstructive sleep apnea. J Clin Endocrinol Metab 2002;87:3394–8.
55. Gonçalves MA, Guilleminault C, Ramos E, Palha A, Paiva T. Erectile dysfunction, obstructive sleep apnea syndrome and nasal CPAP treatment. Sleep Med 2005;6:333–9.
56. Budweiser S, Enderlein S, Jörres RA, et al. Sleep apnea is an independent correlate of erectile and sexual dysfunction. J Sex Med 2009;6:3147–57.
57. Budweiser S, Luigart R, Jörres RA, et al. Long-term changes of sexual function in men with obstructive sleep apnea after initiation of continuous positive airway pressure. J Sex Med 2013;10:524–31.
58. Andersen ML, Alvarenga TF, Mazaro-Costa R, Hachul HC, Tufik S. The association of testosterone, sleep, and sexual function in men and women. Brain Res 2011;1416:80–104.

59. Maia LO, Júnior WD, Carvalho LS, et al. Association of methamidophos and sleep loss on reproductive toxicity of male mice. Environ Toxicol Pharmacol 2011;32:155–6.
60. Jensen TK, Andersson AM, Skakkebæk NE, et al. Association of sleep disturbances with reduced semen quality: a cross-sectional study among 953 healthy young Danish men. Am J Epidemiol 2013;177:1027–37.
61. Lam JC, Mak JC, Ip MS. Obesity, obstructive sleep apnoea and metabolic syndrome. Respirology 2012;17:223–36.
62. Jokela M, Elovainio M, Kivimaki M. Lower fertility associated with obesity and underweight: the US National Longitudinal Survey of Youth. Am J Clin Nutr 2008;88:886–93.
63. Nguyen RH, Wilcox AJ, Skjaerven R, Baird DD. Men's body mass index and infertility. Hum Reprod 2007;22:2488–93.
64. Hammoud AO, Gibson M, Peterson CM, Meikle AW, Carrell DT. Impact of male obesity on infertility: a critical review of the current literature. Fertil Steril 2008;90:897–904.
65. Jung A, Schuppe HC. Influence of genital heat stress on semen quality in humans. Andrologia 2007;39:203–15.
66. Vincent HK, Innes KE, Vincent KR. Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. Diabetes Obes Metab 2007;9:813–39.
67. Tunc O, Bakos HW, Tremellen K. Impact of body mass index on seminal oxidative stress. Andrologia 2011;43:121–8.
68. Kort HI, Massey JB, Elsner CW, et al. Impact of body mass index values on sperm quantity and quality. J Androl 2006;27:450–2.
69. Lombardo F, Sansone A, Romanelli F, Paoli D, Gandini L, Lenzi A. The role of antioxidant therapy in the treatment of male infertility: an overview. Asian J Androl 2011;13:690–7.
70. Bray I, Gunnell D, Davey Smith G. Advanced paternal age: how old is too old? J Epidemiol Community Health 2006;60:851–3.
71. Salonia A, Matloob R, Saccà A, et al. Are Caucasian-European men delaying fatherhood? Results of a 7 year observational study of infertile couples with male factor infertility. Int J Androl 2012;35:125–32.
72. Finkelstein EA, Khavjou OA, Thompson H, et al. Obesity and severe obesity forecasts through 2030. Am J Prev Med 2012;42:563–70.
73. Stevens GA, Singh GM, Lu Y, et al; Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Body Mass Index). National, regional, and global trends in adult overweight and obesity prevalences. Popul Health Metr 2012;10:22.